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Transmitted herewith for filing is a Patent application:

Applicant(s): Massimo PORRO

For: "VACCINES FOR PREVENTION OF GRAM-NEGATIVE BACTERIAL
INFECTIONS AND ENDOTOXIN-RELATED DISEASES"

Enclosed are:

- ☒ Specification/28 pages; Claims/53 and Abstract.
- ☒ Ten (10) sheet(s) of Formal Drawing(s)/Figs. 1-6b.
Preliminary Amendment.
- ☒ Declaration, Power of Attorney and Petition.
- ☒ Verified Statement Claiming Small Entity Status.
- ☒ Statement Under 37 CFR §821(f)
- ☒ An assignment of the invention to: BiosYnth s.r.l.
- ☒ Sequence ID List and 3.5" disk.
- ☒ Information Disclosure Statement.
- ☒ Form PTO-1495 w/cited references.

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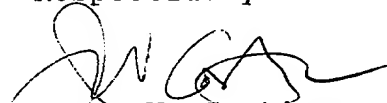
James V. Costigan, Registration No. 25,669
(Typed or printed name of person mailing)

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Respectfully submitted,



James V. Costigan
Registration No. 25,669

MAILING ADDRESS:

HEDMAN, GIBSON & COSTIGAN, P.C.
1185 Avenue of the Americas
New York, NY 10036-2601
(212) 302-8989

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UNITED STATES PATENT APPLICATION

Of: Massimo Porro

For: VACCINES FOR PREVENTION OF
GRAM-NEGATIVE BACTERIAL INFECTIONS AND
ENDOTOXIN-RELATED DISEASES

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VACCINES FOR PREVENTION OF
GRAM-NEGATIVE BACTERIAL INFECTIONS AND
ENDOTOXIN-RELATED DISEASES

5 The present invention is concerned with providing
a vaccine for prevention of bacterial infections caused by
gram-negative bacteria and for the prevention of the
biological effects of homologous endotoxins.

BACKGROUND OF THE INVENTION

10 LPS is the major antigen of gram-negative
bacteria. This material is a glycopospholipid consisting
of an antigenic, variable size, carbohydrate chain
covalently linked to lipid A, the conserved hydrophobic
region structurally defined as N,O-acyl beta-1,6-D-
15 glucosamine 1,4'-bisphosphate. Toxicity of LPS is
expressed by lipid A through the interaction with B-cells
and macrophages of the mammalian immune system, a process
leading to the secretion of proinflammatory cytokines,
mainly TNF, which may have fatal consequences for the host.
20 Lipid A also activates human T-lymphocytes (Th-1) "in
vitro" as well as murine CD4+ and CD8+ T-cell "in vivo", a
property which allows the host's immune system to mount a
specific, anamnestic IgG antibody response to the variable-
size carbohydrate chain of LPS. On these bases, LPS has
25 been recently recognized as a T-cell dependent antigen "in
vivo".

 In order to fully express toxicity, LPS must
retain its supramolecular architecture, through the
association of several units of glycopospholipid monomers
30 forming the lipid A structure. This conformational
rearrangement of the molecule is also fundamental for full
expression of the immunogenic characteristic. Therefore,
dissociation of these intrinsic properties of the molecule
appear to be of crucial interest for proposing the design
35 of LPS-based vaccines related to the prophylaxis of acute
and chronic pathologies due to gram-negative bacterial

infections like meningococcal meningitis, typhoid fever and Helicobacter pylori-induced gastritis.

Sepsis and septic shock are well defined clinical conditions that are caused by bacteria and by LPS which is the endotoxin elaborated by the bacteria responsible for the above mentioned pathologies. The present inventor has described treatment regimens for septic shock which are based on the use of a defined class of peptides that have been demonstrated to be capable of neutralizing LPS in vivo and protecting mammal from septic shock induced by LPS.

The treatment of a subject for septic shock requires that the subject who has symptoms of LPS toxicity be given the peptide when symptoms appear. The peptide is not an immunogenic compound for the production of antibodies to LPS and its use prophylactically will not prevent sepsis which is caused by the bacteria which release LPS. LPS has immunogenic properties but it is too toxic to be used to induce the production of antibodies in a host who is to be protected from the effects of a bacterial infection and from the effects of LPS which is released by certain bacterial infections.

Certain of the peptides are disclosed in U. S. 5,371,186, and information about the basis of the vaccine is disclosed in J. Endotoxin Res. (1997) 4(4)261-272, which is incorporated by reference.

SUMMARY OF THE INVENTION

The applicant has discovered that a vaccine may be prepared by making an endotoxoid that is made by combining LPS free or in conjugate form with a stoichiometric excess of a peptide of the formula:

- (a) $(A)_n$ wherein A is Lysine or Arginine and n is an integer with a minimum value of 7;
- (b) $(AB)_m$ wherein A is Lysine or Arginine and B is a

hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a minimum value of 3; and (c) $(ABC)_p$ wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a minimum value of 2. The peptides of the invention may be terminated independently with a hydrogen atom or any of the naturally occurring amino acids, a fatty acid residue or a carbohydrate residue.

The vaccine is particularly useful for the prevention of gram-negative infections and the effects of endotoxins.

Accordingly, it is a primary object of the invention to provide a method for preparing a vaccine for the prevention of sepsis and septic shock;

It is also an object of the invention to provide a novel vaccine for the prevention of sepsis and septic shock.

It is also an object of the invention to provide a novel vaccine based on an endotoxoid complex of LPS/peptide or conjugated LPS/peptide complex derived from homologous LPS.

It is also an object of this invention to provide novel compositions and methods for the treatment of microbial infections.

These and other objects of the invention will become apparent from the appended specification.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is graph which compares the percent of TNF produced "in vivo" by LPS A1 and various LPS A1 conjugate antigens

detoxified by a peptide which is representative of the class of peptides which may be used in the present invention.

5 FIG. 2a comprises a graph which illustrates the kinetic of serum IgG production in SW mice induced by LPS A1, using IgG which is specific for LPS A1. The filled squares are LPS A1; open squares with the cross are LPS A1/SAEP2 peptide (SAEP2 is: Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys)
10 s - - - - - s
complex (ratio LPS A1/ SAEP2 = 1:250(v/v)); inverted open triangle BSA-LPS A1; filled circle BSA-LPS A1/peptide SAEP2 complex (ratio LPS A1/SAEP2 = 1:250(v/v)). The titers of IgG, represented by the OD value of each symbol were
15 detected by ELISA in the sera pool of each animal group at a standard dilution of 1:200(v/v)

FIG. 2b comprises a graph which is similar to FIG. 2a except that the serum was specific for the carrier protein
20 BSA.

FIG. 3 is a graph which illustrates the lack of toxicity by quantitation of the TNF release in the serum of SW mice 90 min. after s.c. injections (3 weeks apart), of endotoxoid
25 of *N. meningitidis* A1 prepared in different formulations.

FIG.4a shows the kinetic of serum IgG production in SW mice induced by four doses of endotoxoid of *N. meningitidis* A1 injected s.c. three weeks apart. All sera were diluted at
30 1:800(v/v).

FIG.4b shows the kinetic of serum IgG production in CD1 mice induced by four doses of endotoxoid of *Salmonella typhimurium* injected s.c. three weeks apart. All sera were
35 diluted at 1:10,000(v/v).

FIG. 5a is a graph which shows the immunochemical specificity of mouse IgG antibody induced by the endotoxoid A1 prepared from *N. meningitidis* Strain A1.

5

FIG. 5b is a graph which shows the immunochemical specificity of mouse IgG antibody induced by the endotoxoid Ty prepared from *S. enterica* (Seroovar typhimurium).

10 FIG. 6a is a graph which shows the protection against an i.p. challenge of *S. enterica* (Seroovar typhimurium) in CD1 mice immunized either with endotoxoid Ty or homologous/heterologous antigens as controls where the LD₁₀₀ (dose of bacteria killing 100% of the animal population) is 4×10^5 cells.

FIG. 6b is a graph which is the same as FIG. 5a except that it shows the protection against an i.p. challenge of *S. enterica* in CD1 mice immunized either with endotoxoid Ty or
20 homologous/heterologous antigens as controls where the LD₅₀ is 9×10^3 cells.

DETAILED DESCRIPTION OF THE INVENTION

25 The toxic characteristics of LPS may be abrogated without elimination of the antigenic and immunogenic properties of LPS by binding the LPS (via the lipid A moiety) to a peptide as defined in the present application. Typical species of bacteria which produce LPS include *N. meningitidis* Group A,B,C. W135, Y; *E. coli* (especially strain 0157); *Salmonella typhi*; *Salmonella paratyphi*; (A and B) *Shigella flexneri*; non-typeable *Haemophilus influenzae*; *Haemophilus influenzae*, type b; *Helicobacter pylori*; *Chlamydia trachomatis*; *Chlamydia pneumoniae*;
30 *Bordatella pertussis*; *Brucella*; *Legionella pneumophila*;
35

Vibrio cholera (type 01 and non-01); Moraxella catharralis; Pseudomonas aeruginosa; and Klebsiella pneumonia (all species). In particular, the toxicity of structurally different LPS' has been completely abrogated
5 by a lipid A-binding cyclic decapeptide, without affecting the structural integrity of the lipid A moiety and the supramolecular architecture of the antigen. It has been found that different LPS' exhibit an active lipid A moiety with a binding site which can be stoichiometrically
10 saturated in vitro, with high affinity, with a peptide according to the present invention. However, it has been found that for in vivo detoxification of LPS with a peptide according to the present invention, it is necessary to use an excess of peptide with respect to the stoichiometric
15 amount required "in vitro" to sufficiently detoxify LPS for preparing an immunogenic endotoxoid (complex) which will induce antibody formation without any unacceptable toxicity. It is believed that the stoichiometric excess is necessary to significantly stabilize the LPS-peptide
20 complex from the likely antagonistic activity of natural LPS-receptor proteins present on specialized cells of the immune system which bear amino acid sequences similar to that of the peptides used in the present invention.

Generally, the immunogenic compound, or
25 endotoxoid complex of the invention, is prepared by combining LPS, derived from a bacterial source, with from 2-10 to 2-5000, preferably from 10-5000 and especially preferably from 250-2500 times its weight, of a peptide as described herein based on a weight/weight ratio of LPS to
30 the peptide. Higher molecular weight peptides may require a higher ratio of peptide to LPS. It is also possible to first conjugate the LPS with a protein such as bovine serum albumin, tetanus toxoid or diphtheria toxoid or non-toxic diphtheria mutant proteins (CRM197) or outer-membrane
35 proteins (OMP) prior to combining the LPS with the peptide

to form the endotoxoid complex. Generally a ratio of 2:1 of LPS to BSA may be employed in the conjugation procedure to yield a covalent conjugate of LPS:BSA =1 (w/w).

The peptides which may be complexed with the lipid A moiety of LPS include linear or cyclic peptides having units of the formula:

(a) $(A)_n$ wherein A is Lysine or Arginine and n is an integer with a minimum value of 7;

(b) $(AB)_m$ wherein A is Lysine or Arginine and B is a hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a minimum value of 3; and

(c) $(ABC)_p$ wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a minimum value of 2. The peptides of the invention may be terminated independently with a hydrogen atom or any of the naturally occurring amino acids, a fatty acid residue or a carbohydrate residue. In addition the retroinverted peptides, the enantiomer amino acid sequences (all -D amino acids in the sequence), the diastereomer amino acid sequences (-D and -L amino acids in the sequence), and the peptide sequences in which the amino acids are inverted with respect to their original position in the sequence which are based on the peptides described herein may also be employed.

The preferred peptides for use in the invention will also have a ratio of aliphatic cationic amino acids to hydrophobic amino acids ($R_{C/H}$) of at least 0.5 and within the range of about 0.5 to 10.0 which is computed by using the solvent parameter values only for those amino acids which are present in the peptides which have a solvent parameter value equal to or greater than +1.5kcal/mol

(lysine and arginine) and -1.5kcal/mol (valine, isoleucine, leucine, tyrosine, phenylalanine and tryptophane) as measured according to Levitt, J. Mol. Biol. 104,59 (1976), which is incorporated by reference.

5 The peptide sequence for use in preparing a vaccine according to the invention will preferably comprise six to ten amino acid residues containing a minimum of three aliphatic cationic amino acids, with a ratio of aliphatic cationic amino acids to hydrophobic
10 amino acids of equal to or greater than 0.5 (R_c/h wherein c is the number of cationic amino acids in the peptide and h is the number of hydrophobic amino acids in the peptide). This ratio is believed to be the minimum although sequences of ten amino acids with a ratio (R_c/h) equal to or greater
15 than 1.0 are optimal for expression of biological activity.

 The peptide units which are represented by formula (a), (b) and (c) represent discrete peptides which will also potentiate antibiotics as well as peptides which will bind and neutralize endotoxin in the LAL test and
20 which include as a part of their structure units of formula (a), (b) and (c), in addition to other amino acids, are included within the peptides which comprise the invention.

 The preferred minimum values for n , m and p have been determined experimentally on the basis of the
25 observation that when the peptide is linear, it should have at least 7 amino acid units and when said peptide is cyclic or a polymer having several cycles, i.e. 2 to 6 cycles, it will have a ring structure that has a minimum of 6 amino acid units; said peptides having a ratio of aliphatic
30 cationic amino acids to hydrophobic amino acids which is equal to or greater than 0.5.

 When the peptides are of the formula $(A)_n$, $(AB)_m$ or $(ABC)_p$, i.e. when these formulas do not represent units of a larger peptides, n will be from 7 to 500 and
35 preferably from 7 to 16; m will be from 3 to 200 and

preferably from 4 to 20 and p will be from 2 to 100 and preferably from 4 to 20.

Examples of the peptides are listed below.

- 5 (Lys)₁₀ (SEQ ID NO: 1);
(Lys)₃₀ (SEQ ID NO: 2);
(Lys)₄₃₄ (SEQ ID NO: 3);
(Lys-Asp)₅ (SEQ ID NO: 4);
(Lys-Phe)₅ (SEQ ID NO: 5);
- 10 Lys-Phe-Leu-Lys-Lys-Thr-Leu (SEQ ID NO: 6);
(Lys-Phe-Leu)₂-Lys (SEQ ID NO: 7);
(Lys-Phe-Leu)₃-Lys (SEQ ID NO: 8);
(Arg-Tyr-Val)₃ (SEQ ID NO: 9);
(Lys-Phe-Phe)₃-Lys (Seq ID NO: 10);
- 15 (Lys-Leu-Leu)₃ (SEQ ID NO: 11);
(Lys)₆(Phe-Lys)₂ (SEQ ID NO: 12);
Cys-(Lys)₅-Cys
s-----s (SEQ ID NO: 13);
Cys-Lys-Phe-Lys-Lys-Cys
20 s-----s (SEQ ID NO: 14);
Lys-Phe-Lys-Cys-Lys-Phe-Lys-Phe-Lys-Cys
s-----s (SEQ ID NO: 15);
Lys-Leu-Lys-Cys-Lys-Leu-Lys-Leu-Lys-Cys
s-----s (SEQ ID NO: 16);
- 25 Arg-Thr-Arg-Cys-Arg-Phe-Lys-Arg-Arg-Cys
s-----s (SEQ ID NO: 17);
Lys-Cys-(Lys-Phe-Lys)₂-Cys-Lys
s-----s (SEQ ID NO: 18);
Cys-(Lys)₄-(Phe)₄-Cys
30 s-----s (SEQ ID NO: 19);
Cys-(Lys-Phe-Leu)₃-Lys-Cys
s-----s (SEQ ID NO: 20);
Val-Lys-Ala-Leu-Arg-Val-Arg-Arg-Leu (SEQ ID NO: 21);
Lys-Ser-Leu-Ser-Leu-Lys-Arg-Leu-Thr-Tyr-Arg (SEQ ID NO: 22);
- 35 Lys-Val-Arg-Lys-Ser-Phe-Phe-Lys-Val (SEQ ID NO: 23);
Phe-Leu-Lys-Pro-Gly-Lys-Val-Lys-Val (SEQ ID NO: 24);
Lys-Glu-Leu-Lys-Arg-Ile-Lys-Ile (SEQ ID NO: 25);
Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu (SEQ ID NO: 26);
Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu-Lys (SEQ ID NO: 27);

Lys-Arg-Leu-Lys-Trp-Lys-Tyr-Lys-Gly-Lys-Phe (SEQ
ID NO: 28); and
Cys-Gln-Ser-Trp-Lys-Ser-Ser-Glu-Ile-Arg-Cys-Gly-Lys
s-----s (SEQ ID NO:
5 29).
Cys-Lys-Phe-Leu-Lys-Lys-Cys
s - - - - - s (SEQ ID NO:30)
10 Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys (SEQ ID NO:31)
s - - - - - s
Lys-Phe-Leu-Lys-Lys-Thr (SEQ ID NO: 32)
Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr-Lys
15 s - - - - - s (SEQ ID NO: 33)
Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr
s - - - - - s (SEQ ID NO: 34)
20 Ile-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys
s - - - - - s (SEQ ID NO: 35)
Ile-Lys-Thr-Lys-Lys-Phe-Leu-Lys-Lys-Thr (SEQ ID NO: 36)
25 Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu-Lys (SEQ ID NO: 37)
Lys-Phe-Leu-Lys-Phe-Leu-Lys (SEQ ID NO: 38)
30 Arg-Tyr-Val-Arg-Tyr-Val-Arg-Tyr-Val (SEQ ID NO: 39)
Lys-Phe-Phe-Lys-Phe-Phe-Lys-Phe-Phe (SEQ ID NO: 40)
Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu (SEQ ID NO:41)
35 (Lys)⁶Phe-Leu-Phe-Leu (SEQ ID NO:42)
Cys-Lys-Phe-Lys-Phe-Lys-Phe-Lys-Phe-Cys
s-----s (SEQ ID NO: 43
40 Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu-Lys (SEQ ID NO: 44)
Lys-Arg-Leu-Lys-Trp-Lys-Tyr-Lys-Gly-Lys-Phe (SEQ ID NO: 45)

45 The peptides for use in the present invention may
be synthesized by classical methods of peptide chemistry
using manual or automated techniques as well as by DNA
recombinant technology. The synthetic procedure comprises
50 solid phase synthesis by Fmoc chemistry, cleavage (TFA

95%+Et-(SH)₂ 5%), followed by vacuum evaporation. Thereafter, the product is dissolved in 10% acetic acid, extracted with ether, concentrated at 0.1 mg/ml at pH of 6.0-7.5. Stirring under filtered air followed for 1 to 6
5 hours in case of the Cysteine-containing peptides and finally desalting by reverse phase chromatography is carried out.

A particular automated method of preparing peptides for use in the present invention is based on the
10 use of an automatic synthesizer (Milligen Mod.9050 (MILLIPORE, Burlington, MA) on a solid phase support of polyamide/Kieselguhr resin (2.0g). The amino acids used in the synthesis of the peptide analogs are Fmoc-aa-Opfp derivatives (9-Fluorenylmethylcarbonyl-aa-O-
15 pentafluorophenyl ester) of each amino acid(aa) involved in the considered sequences using 0.8 mol of each amino acid to sequentially form the peptide.

Each cycle of synthesis may be performed at room temperature (20°C) and involves the following steps of
20 reaction:

Step 1 - Deprotection

The first aa Fmoc-protected at the amino group, was treated with a 20% solution of piperidine for 7 minutes in order to remove the Fmoc alpha-protecting group. Washing with
25 dimethylformamide followed for 12 minutes to remove all traces of piperidine. Deprotection and washing were run

continuously through the column containing the resin by means of a pump at a flow of 5ml/min.

Step 2 - Activation of the Fmoc-aa-Opfp derivative

5 The amino and carboxy-protected amino acid due, according to the desired sequence, was activated after its dissolution in 5 ml of dimethylformamide, by a catalytic amount of hydroxybenzotriazol (0.5 ml of a 5% w/v solution in dimethylformamide).

Step 3 - Acylation

10 The activated and protected Fmoc-aa-Opfp derivative was then recycled for 30 minutes through the column by the pump at 5ml/min in order to obtain coup[ling of the introduced aa at the alpha-amino group (previously deprotected as reported in Step 1) of the amino acid preceding the new one
15 in the desired sequence.

Step 4 - Washing

Washing of the matrix in the column followed by dimethylformamide for 2 minutes at 5 ml/min before a new cycle began.

20 At the completion of the synthesis, the peptide on the resin support was cleaved by 95% Trifluoroacetic acid (TFA) with 5% ethane dithiol as a scavenger, if Cysteine residues were present in the aa sequence, at room temperature for 2 hours. After separation of the cleaved
25 peptide from the resin by filtration, the solution was concentrated by vacuum evaporation to dryness. The collected solid residue was then solubilized in 10% acetic acid at a concentration of 10-20 mg/ml and several extractions by diethyl ether followed (six to eight
30 extractions with half the volume of the peptide solution) in order to remove the scavenger Ethane dithiol. The peptide solution was then neutralized by 0.1 N ammonium hydroxide and adjusted to the concentration of roughly 0.1 mg/ml. The solution was then stirred under air for 1 to 6
35 hours in order to obtain the selective oxidation of the two

sulfhydryl groups belonging to the Cys residues of the sequence. In this way, only monomeric oxidized peptides were obtained with no traces of polymeric material. The solution of oxidized peptide was then desalted by reverse-
5 phase chromatography on SEP-PAK C-18 cartridges (MILLIPORE) and finally freeze dried. The products were analyzed by high-performance liquid chromatography (HPLC) analysis as well as by chemical analysis of the synthetic structures.

Fast atom bombardment may be used to confirm the
10 calculated mass of the peptides.

The vaccines may be administered parenterally, preferably subcutaneously using well known pharmaceutical carriers or inert diluents such as water for injection, sterile normal saline and the like.

15 The LPS and the peptide may be reacted by combining sterile aqueous solutions of the LPS and the peptide followed by incubation for 15 min. to six hours at temperature from 25°C to 40°C. Generally, the effective amount of the endotoxoid complex is from 0.1µg to 50µg/kg
20 of body weight for a mammal. The endotoxoid complex may be employed in humans and in veterinary practice to prevent sepsis and the toxic effects of endotoxin related shock caused by bacterial infections wherein the causative organism elaborates endotoxin. The endotoxoid complex may
25 be administered prophylactically as a vaccine by giving one or more doses to a subject until a protective level of antibodies is detected by the following test: Enzyme Linked Immunoassay (ELISA) or any other clinically acceptable immunoassay.

30 Generally, a vaccination regimen may comprise an initial dose of the vaccine followed by from one to four booster inoculations given at intervals of two to four weeks.

The particular dose of a particular endotoxoid
35 complex may be varied within or without the range that is

specified herein depending on the particular host. Those who are skilled in the art may ascertain the proper dose using standard procedures. The vaccine of the invention may be monovalent in that it contains one endotoxoid complex
5 derived from the LPS obtained from one species of bacteria or it may be polyvalent and contain a plurality of endotoxoid complexes made from LPS which is obtained from different species of bacteria. The endotoxoid complex may also be administered as a part of a multicomponent vaccine
10 such as diphtheria-pertussis-tetanus (DPT) (Tri-immunol, Lederle Laboratories) or diphtheria-pertussis-tetanus-haemophilus (Tetraimmune, Lederle Laboratories). In such cases, an effective amount of the endotoxoid complex or complexes may be combined with the multicomponent vaccine
15 in order to simultaneously induce multiple antibodies in a host.

The invention also includes the combined administration of the vaccine of the invention with an effective amount of an antibiotic and/ or a peptide as
20 described above to simultaneously treat a gram-negative infection and provide an immunizing dose of the vaccine. The amounts of the peptide to be administered are described in U.S. 5,589,459 and the combined antibiotic-peptide therapy is described in Serial No 08/456,112, both of which
25 are incorporated by reference.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

EXAMPLE

30

PREPARATION OF ANTIGENS

LPS from N. meningitidis A1, BB431, 44/77 and purified N. meningitidis LPS A1 were prepared and
35 characterized according to the method of Gu and Tsai,

Infect. Immun. (1993); 61:1873-1879, which is incorporated by reference. The cyclic peptide, described herein by as SAEP2 was synthesized on solid phase, oxidized and characterized according to the methods set forth in Science
5 (1993): 259:361-365, which is incorporated by reference.

The covalent conjugate BSA-LPS A1 was prepared as follows: LPS A1 (5mg/ml) in PBS., containing two reactive moles of amino group per mole of oligosaccharide-lipid A monomer, was transformed to the mono-succinimidyl ester by
10 incubation (60min at room temperature) with the bis-succinimidyl ester of adipic acid (0.7mg/ml) in dimethylsulfoxide (DMSO), according to the procedure reported for amino-activated bacterial capsular oligosaccharides in Mol Immunol (1985):22:907-919.

15 The LPS A1 derivative had more than 98% of the amino groups contained in its structure transformed into highly reactive ester groups as determined by the trinitrobenzene sulfonic acid (TNBS) reaction. The ester-derivative of LPS A1 was then mixed with a sodium bicarbonate solution
20 pH = 8.0 containing 0.9mg/ml of BSA. The stoichiometry of the reagents is equivalent to a molar ratio between the amino groups of BSA:monoester groups of LPS A1=2. The solution was stirred for 4 hours at room temperature and the BSA-LPS A1 conjugate was recovered by precipitation
25 with ethanol (60%v/v final concentration), solubilized in 0.1 M sodium bicarbonate and finally purified using gel chromatography (Sephacrose, Pharmacia) sterile filtered using a 0.22µm membrane and freeze dried. The conjugate is consistent with a ratio of BSA:LPS A1 =1(w/w).

30

PREPARATION OF VACCINES

Complexes of the SAEP2 peptide with both LPS A1 and BSA-LPS A1 conjugate were prepared by combining sterile solutions
35 of LPS A1 or BSA:LPS A1 with a 0.1 to 5% (w/w) solution of

SAEP2 peptide at a ratio of LPS/SAEP2 peptide of 1:250(w/w). The solutions were incubated for 30min. at 37°C. Sodium merthiolate 0.01%(w/v) was added as a preservative and the products were stored at 4°C. The immunizing dose of LPS A1 was in the range of 0.5-5µg in 0.2ml, in the formulations of vaccine tested.

TESTING

The safety of the LPS A1/SAEP2 peptide complex was confirmed "in vitro" by the lack of pyrogenic activity tested by Lipid A-induced LAL clotting and "in vivo" by the determination of TNF (the mediator of toxicity) titers induced by the vaccines in mice (strains SW and CD1). TNF was inhibited by 90% when the LPS A1/SAEP2 peptide complex (1:250w/w ratio) was employed and TNF was inhibited by 98% when the conjugate BSA-LPS A1/SAEP2 peptide complex (1:250w/w ratio) was employed. The results for the various TNF determinations are shown in FIG. 1.

The immunogenicity of the LPS A1/SAEP2 peptide complex was determined by comparing the kinetic pattern of the IgG antibodies specific for LPS A1 which are induced by LPS A1; LPS A1/SAEP2 peptide complex; and BSA-LPS A1; and BSA-LPS A1/SAEP2 peptide complex. The results are shown in FIG. 2a and FIG. 2b. The test data show that immunogenicity of endotoxin A1 (LPS A1/SAEP2 peptide complex) is comparable to that induced by the conjugates BSA-LPS A1 peptide complex. Therefore, toxicity and immunogenicity have been dissociated in an endotoxoid.

EXAMPLE 2

LPS from N. meningitidis group A (LPS A1) and Salmonella enterica (serotype typhimurium, LPS Ty) were used to prepare vaccines from two groups of structurally

different LPS (R- and S-like chemotype) that originated from clinical isolates of extracellular and intracellular Gram-negative bacteria, respectively. Both LPS' exhibit an active lipid A moiety with a binding site which is stoichiometrically saturated "in vitro", with high affinity, by a synthetic cyclic peptide (SAEP2) which exemplifies the peptides of the invention) For "in vivo" testing, peptide complexes were prepared at a ratio of (a) 1:250; 1:1000 and 1:2500 of LPS A1:peptide and LPS Ty:peptide.

The two endotoxoids (A1 and Ty) were completely non-toxic with respect to LPS, as demonstrated by the level of TNF systemically released in mice after four injections of the antigens (FIG. 3). Comparable results were obtained in rabbits by using a hemorrhagic necrosis test or Schwartzman reaction.

Outbred mice were immunized subcutaneously, three weeks apart, by plain LPS A1 and LPS Ty in parallel with the homologous endotoxoids prepared by complex formation with a cyclic peptide (SAEP2 was used as an example). Serum immune response was assayed for specific anti-LPS IgG isotype antibodies, two weeks following each administration. LPS A1 and LPS Ty have shown a minimum immunogenic activity at the dose of 5 ug/mouse. The homologous endotoxoids have expressed, at a dose ten times lower (0.5µg), an immunogenic activity comparable to that obtained with a dose of 5µg of plain LPS.

To explain this observation, it is hypothesized that there is a downregulating activity of TNF on T-cells. For this purpose, mice were immunized with plain LPS A1, whose toxicity was abrogated by the previous administration of a characterized anti-TNF monoclonal antibody. Although the toxicity of plain LPS A1 was abrogated by the anti-TNF treatment (Fig. 3), no significant increase in the immunogenicity of LPS was detected, in contrast to the

homologous endotoxoid (FIG. 4a), suggesting that while TNF is the recognized mediator of LPS toxicity, it is not significantly involved in the immunogenic activity of LPS.

The endotoxoid-induced IgG antibodies were specific for the core oligosaccharide chain of LPS A1 and for the O-saccharide chain of LPS Ty respectively (Fig. 5a and Fig. 5b). The endotoxoids were functional in fixing and activating homologous and heterologous species of complement and were completely protective in a significant model of salmonella infection (FIG. 6a and FIG. 6b). Furthermore, the endotoxoid-induced IgG antibodies were able to passively protect the animals from the endotoxemic effects, detectable by serum TNF release, of a systemic challenge by homologous LPS (Table I).

TABLE I

Effect of anti LPS A1 polyclonal IgG antibodies on the inhibition of serum TNF production in CD1 mice challenged i.v. with homologous *N. meningitidis* A1 LPS and heterologous *E. coli* 055 B5 LPS. Data represent mean of two independent experiments with 5 mice/group. IgG anti LPS A1 were injected i.v. 30 minutes before either *N. meningitidis* A1 LPS or *E. coli* 055:B5 LPS i.v. challenge.

| CHALLENGE | | | |
|-----------|----------------|---------------|---------------|
| Mice | IgG antiLPS A1 | LPS A1 | LPS B5 |
| 10 | saline | 6,110+/-2,062 | 2,371+/-1,471 |
| 10 | 0.25 | 1,579+/-591 | 2,068+/-1,864 |

(inhibition 74%, p<0.01)

These experimental results show the protective activity, in a mammalian host, of a bacterial endotoxoid originating from LPS of either extracellular or intracellular gram-negative pathogens.

While certain preferred and alternative

embodiments of the invention have been set forth for purposes of disclosing the invention, modifications to the disclosed embodiments may occur to those who are skilled in the art. Accordingly, the appended claims are intended to
5 cover all embodiments of the invention and modifications thereof which do not depart from the spirit and scope of the invention.

I Claim:

1. A vaccine for preventing gram negative infections and the effects of endotoxins which comprises a complex
5 obtained by combining LPS free or in conjugate form with a stoichiometric excess of a peptide of the formula:
(a) $(A)_n$ wherein A is Lysine or Arginine and n is an integer with a minimum value of 7;
(b) $(AB)_m$ wherein A is Lysine or Arginine and B is a
10 hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a minimum value of 3; and
(c) $(ABC)_p$ wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids
15 which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a minimum value of 2.
- 20 2. A vaccine as defined in claim 1 wherein the peptide is a linear or cyclic peptides having units of the formula:
(a) $(A)_n$ wherein A is Lysine or Arginine and n is an integer with a value of 7 to 16;
(b) $(AB)_m$ wherein A is Lysine or Arginine and B is a
25 hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a value of 4 to 20; and
(c) $(ABC)_p$ wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids
30 which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a value of 4 to 20.
- 35 3. A vaccine as defined in claim 1 wherein the LPS is

derived from N. meningitidis.

4. A vaccine as defined in claim 1 wherein the LPS is derived from Salmonella typhi.

5 5. A vaccine as defined in claim 1 where the amount of peptide is from 2-10 to 2-5000 times the weight of the LPS.

6. A vaccine as defined in claim 1 wherein the peptide has units of the formula $(AB)_m$.

10

7 A vaccine as defined in claim 1 wherein the peptide has units of the formula $(ABC)_p$.

8. A vaccine as defined in claim 1 wherein the peptide is
15 of the formula:
(Lys)₁₀. (SEQ ID NO: 1)

9. A vaccine as defined in claim 1 wherein the peptide is
of the formula:
20 (Lys-Glu)₅. (SEQ ID NO: 4)

10. A vaccine as defined in claim 1 wherein the peptide is
of the formula:
(Lys-Phe)₅. (SEQ ID NO: 5)

25

11. A vaccine as defined in claim 1 wherein the peptide is
of the formula:
Lys-Phe-Leu-Lys-Lys-Thr-Leu. (SEQ ID NO: 6)

30 12. A vaccine as defined in claim 1 wherein the peptide is
of the formula:
(Lys-Phe-Leu)₂-Lys. (SEQ ID NO: 7)

13. A vaccine as defined in claim 1 wherein the peptide is
35 of the formula:

(Lys-Phe-Leu)₃-Lys. (SEQ ID NO: 8)

14. A vaccine as defined in claim 1 wherein the peptide is of the formula:

(Arg-Tyr-Val)₃. (SEQ ID NO: 9)

5

15. A vaccine as defined in claim 1 wherein the peptide is of the formula:

(Lys-Phe-Phe)₃-Lys. (Seq ID NO: 10)

10 16. A vaccine as defined in claim 1 wherein the peptide is of the formula:

(Lys-Leu-Leu)₃ (SEQ ID NO: 11)

15 17. A vaccine as defined in claim 1 wherein the peptide is of the formula:

(Lys)₆(Phe-Lys)₂. (SEQ ID NO: 12)

18. A vaccine as defined in claim 1 wherein the peptide is of the formula:

20 Cys-(Lys)₅-Cys
s-----s. (SEQ ID NO: 13)

19. A vaccine as defined in claim 1 wherein the peptide is of the formula:

25 Cys-Lys-Phe-Lys-Lys-Cys
s-----s. (SEQ ID NO: 14)

20. A vaccine as defined in claim 1 wherein the peptide is of the formula:

30 Lys-Phe-Lys-Cys-Lys-Phe-Lys-Phe-Lys-Cys
s-----s. (SEQ ID NO: 15)

21. A vaccine as defined in claim 1 wherein the peptide is of the formula:

35 Lys-Leu-Lys-Cys-Lys-Leu-Lys-Leu-Lys-Cys
s-----s. (SEQ ID NO: 16)

22. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Arg-Thr-Arg-Cys-Arg-Phe-Lys-Arg-Arg-Cys
s-----s. (SEQ ID NO: 17)

5

23. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Lys-Cys-(Lys-Phe-Lys)₂-Cys-Lys
s-----s. (SEQ ID NO: 18)

10

24. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Cys-(Lys)₄-(Phe)₄-Cys
s-----s. (SEQ ID NO: 19)

15

25. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Cys-(Lys-Phe-Leu)₃-Lys-Cys
s-----s. (SEQ ID NO: 20)

20

26. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Val-Lys-Ala-Leu-Arg-Val-Arg-Arg-Leu (SEQ ID NO: 21)

25 27. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Lys-Ser-Leu-Ser-Leu-Lys-Arg-Leu-Thr-Tyr-Arg (SEQ ID NO: 22)

28. A vaccine as defined in claim 1 wherein the peptide is of the formula:

Lys-Val-Arg-Lys-Ser-Phe-Phe-Lys-Val (SEQ ID NO: 23)

29. A vaccine as defined in claim 1 wherein the peptide is of the formula:

35 Phe-Leu-Lys-Pro-Gly-Lys-Val-Lys-Val (SEQ ID NO: 24)

30. A vaccine as defined in claim 1 wherein the peptide is

of the formula:

Lys-Asp-Leu-Lys-Arg-Ile-Lys-Ile (SEQ ID NO: 25)

31. A vaccine as defined in claim 1 wherein the peptide is
5 of the formula:

Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu (SEQ ID NO: 26)

32. A vaccine as defined in claim 1 wherein the peptide is
10 of the formula:

Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu-Lys (SEQ ID NO: 27)

33. A vaccine as defined in claim 1 wherein the peptide is
15 of the formula:

Lys-Arg-Leu-Lys-Trp-Lys-Tyr-Lys-Gly-Lys-Phe (SEQ ID NO:28)

34. A vaccine as defined in claim 1 wherein the peptide is
20 of the formula:

Cys-Gln-Trp-Lys-Ser-Ser-Asp-Ile-Arg-Cys-Gly-Lys
S-----S (SEQ ID NO: 29)

35. A vaccine as defined in claim 1 wherein the peptide is
25 of the formula:

Cys-Lys-Phe-Leu-Lys-Lys-Cys
S-----S (Seq ID NO: 30)

36. A vaccine as defined in claim 1 wherein the peptide is
30 of the formula:

Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys (SEQ ID NO:31)
S - - - - - S

37. A vaccine as defined in claim 1 wherein the peptide is
35 of the formula:

Lys-Phe-Leu-Lys-Lys-Thr (SEQ ID NO: 32)

38. A vaccine as defined in claim 1 wherein the peptide is
40 of the formula:

Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr-Lys
S - - - - - s (SEQ ID NO: 33)

- 5 39. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

10 Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr
S - - - - - s (SEQ ID NO: 34)

40. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

15 Ile-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys
S - - - - - s (SEQ ID NO: 35)

41. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

20 Ile-Lys-Thr-Lys-Lys-Phe-Leu-Lys-Lys-Thr (SEQ ID NO: 36)

42. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

25 Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu-Lys (SEQ ID NO: 37)

43. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

30 Lys-Phe-Leu-Lys-Phe-Leu-Lys (SEQ ID NO: 38)

44. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

35 Arg-Tyr-Val-Arg-Tyr-Val-Arg-Tyr-Val (SEQ ID NO: 39)

45. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

40 Lys-Phe-Phe-Lys-Phe-Phe-Lys-Phe-Cys (SEQ ID NO: 40)

46. A vaccine as defined in claim 1 wherein the peptide is
of the formula:

45

Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu (SEQ ID NO:41)

47. A vaccine as defined in claim 1 wherein the peptide is of the formula:

5

(Lys)₆Phe-Leu-Phe-Leu (SEQ ID NO:42)

48. A vaccine as defined in claim 1 wherein the peptide is of the formula:

10

Cys-Lys-Phe-Lys-Phe-Lys-Phe-Lys-Phe-Cys
s-----s (SEQ ID NO: 43)

49. A vaccine as defined in claim 1 wherein the peptide is of the formula:

15

Lys-Trp-Lys-Ala-Gln-Lys-Arg-Phe-Leu-Lys (SEQ ID NO: 44)

50. A vaccine as defined in claim 1 wherein the peptide is of the formula:

20

Lys-Arg-Leu-Lys-Trp-Lys-Tyr-Lys-Gly-Lys-Phe (SEQ ID NO: 45)

51. A method for the preparation of a vaccine for prevention of gram-negative infections and the effects of endotoxins, said method comprising combining LPS with a stoichiometric excess of a peptide of the formula:

25

(a) (A)_n wherein A is Lysine or Arginine and n is an integer with a minimum value of 7;

30

(b) (AB)_m wherein A is Lysine or Arginine and B is a hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a minimum value of 3; and

35

(c) (ABC)_p wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a minimum value of 2.

40

52. A vaccine as defined in claim 1 which is combined or administered with other vaccine components.

53. A vaccine as defined in claim 1 which contains an LPS peptide complex derived from more than one species of bacteria.

ABSTRACT

A vaccine is disclosed which is useful for protecting a host from Gram negative infections and the effects of endotoxin, therefore preventing sepsis and septic shock. The vaccine is prepared by combining LPS free or in conjugate form with a stoichiometric excess of a peptide of the formula:

- (a) $(A)_n$ wherein A is Lysine or Arginine and n is an integer with a minimum value of 7;
- (b) $(AB)_m$ wherein A is Lysine or Arginine and B is a hydrophobic amino acid selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; m is an integer with a minimum value of 3; and
- (c) $(ABC)_p$ wherein A is a cationic amino acid which is Lysine or Arginine; B and C are hydrophobic amino acids which may be the same or different and are selected from the group consisting of Valine, Leucine, Isoleucine, Tyrosine, Phenylalanine and Tryptophan; p is an integer with a minimum value of 2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Porro, Massimo

(ii) TITLE OF INVENTION: VACCINES FOR PREVENTION OF GRAM-
NEGATIVE BACTERIAL INFECTIONS AND ENDOTOXIN RELATED DISEASES

(iii) NUMBER OF SEQUENCES: 45

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Hedman, Gibson & Costigan
(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage
(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: Word Perfect 5.1

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Costigan, James V.
(B) REGISTRATION NUMBER: 25,669
(C) REFERENCE/DOCKET NUMBER: 576-008

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 302-8989
(B) TELEFAX: (212) 302-8998

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
1 5 10

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
1 5 10
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
15 20
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
25 30

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 434 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
1 5 10
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
15 20
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
25 30
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
35 40
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
45 50
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
55 60
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
65 70
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
75 80
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
85 90
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
95 100
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
105 110
Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys
115 120

[illegible]

| | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys |
| | | | | 405 | | | | | | 410 |
| Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys |
| | | | | 415 | | | | | | 420 |
| Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys | Lys |
| | | | | 425 | | | | | | 430 |
| Lys | Lys | Lys | Lys | | | | | | | |

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Asp | Lys | Asp | Lys | Asp | Lys | Asp | Lys | Asp |
| 1 | | | | 5 | | | | | 10 |

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Phe | Lys | Phe | Lys | Phe | Lys | Phe | Lys | Phe |
| 1 | | | | 5 | | | | | 10 |

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

| | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|
| Lys | Phe | Leu | Lys | Lys | Thr | Leu |
| 1 | | | | 5 | | |

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Phe Leu Lys Phe Leu Lys
1 5

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Phe Leu Lys Phe Leu Lys Phe Leu Lys
1 5 10

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Tyr Val Arg Tyr Val Arg Tyr Val
1 5

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys Phe Phe Lys Phe Phe Lys Phe Phe Lys
1 5 10

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Leu Leu Lys Leu Leu Lys Leu Leu
1 5

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Lys Lys Lys Lys Lys Lys Phe Lys Phe Lys
1 5 10

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Lys Cys Lys Cys Lys Cys Lys Cys Lys Cys
1 5 10

(15) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Cys Lys Phe Lys Lys Cys
1 5

(16) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Lys Phe Lys Cys Lys Phe Lys Phe Lys Cys
1 5 10

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid

(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:16:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Leu | Lys | Cys | Lys | Leu | Lys | Leu | Lys | Cys |
| 1 | | | | 5 | | | | | 10 |

(18) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:17:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Thr | Arg | Cys | Arg | Phe | Lys | Arg | Arg | Cys |
| 1 | | | | 5 | | | | | 10 |

(19) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:18:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Cys | Lys | Phe | Lys | Lys | Phe | Lys | Cys | Lys |
| 1 | | | | 5 | | | | | 10 |

(20) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:19:

| | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Lys | Lys | Lys | Lys | Phe | Phe | Phe | Phe | Cys |
| 1 | | | | 5 | | | | | 10 |

(21) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Cys Lys Phe Leu Lys Phe Leu Lys Phe Leu Lys Cys
 1 5 10

(22) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Val Lys Ala Leu Arg Val Arg Arg Leu

(23) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Ser Leu Ser Leu Lys Arg Leu Thr Tyr Arg

(24) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Val Arg Lys Ser Phe Phe Lys Val

(25) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Phe Leu Lys Pro Gly Lys Val Lys Val

(26) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids

(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Lys Glu Leu Lys Arg Ile Lys Ile

(27) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Lys Trp Lys Ala Gln Lys Arg Phe Leu

(28) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys

(29) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Lys Arg Leu Lys Trp Lys Tyr Lys Gly Lys Phe 28)

(30) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Cys Gln Ser Trp Lys Ser Ser Glu Ile Arg Cys Gly Lys
1 5 10

(31) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Cys Lys Phe Leu Lys Lys Cys
1 5

(32) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Lys Thr Lys Cys Lys Phe Leu Lys Lys Cys
1 5 10

(33) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Lys Phe Leu Lys Lys Thr

(34) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Cys Lys Lys Leu Phe Lys Cys Lys Thr Lys
1 5 10

(35) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids

(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Cys Lys Lys Leu Phe Lys Cys Lys Thr
1 5

(36) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Ile Lys Thr Lys Cys Lys Phe Leu Lys Lys Cys
1 5 10

(37) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Ile Lys Thr Lys Lys Phe Leu Lys Lys Thr

(38) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Ile Lys Phe Leu Lys Phe Leu Lys Phe Leu Lys

(39) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Lys Phe Leu Lys Phe Leu Lys

(40) INFORMATION FOR SEQ ID NO:39

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Arg Tyr Val Arg Tyr Val Arg Tyr Val

(41) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Lys Phe Phe Lys Phe Phe Lys Phe Phe

(42) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Ile Lys Phe Leu Lys Phe Leu Lys Phe Leu

(43) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Lys Lys Lys Lys Lys Lys Phe Leu Phe Leu

(44) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Cys Lys Phe Lys Phe Lys Phe Lys Phe Cys
1 5 10

(45) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Lys Trp Lys Ala Gln Lys Arg Phe Leu Lys

(46) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Lys Arg Leu Lys Trp Lys Tyr Lys Gly Lys Phe

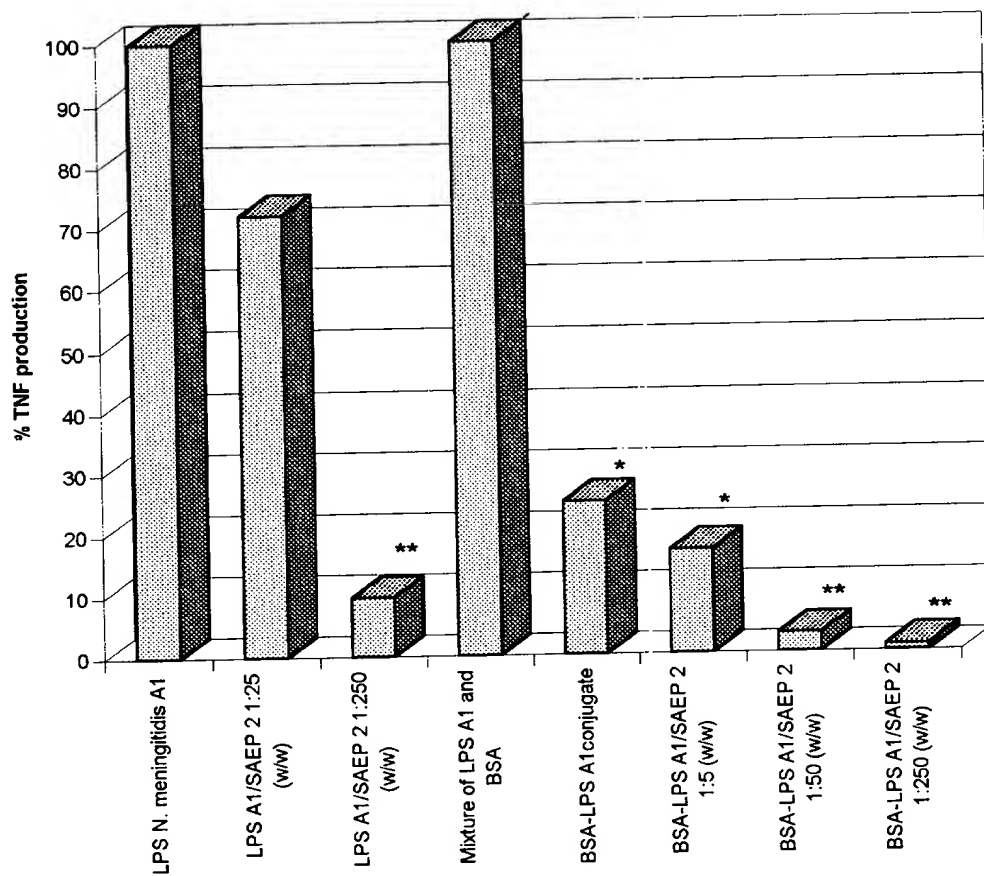
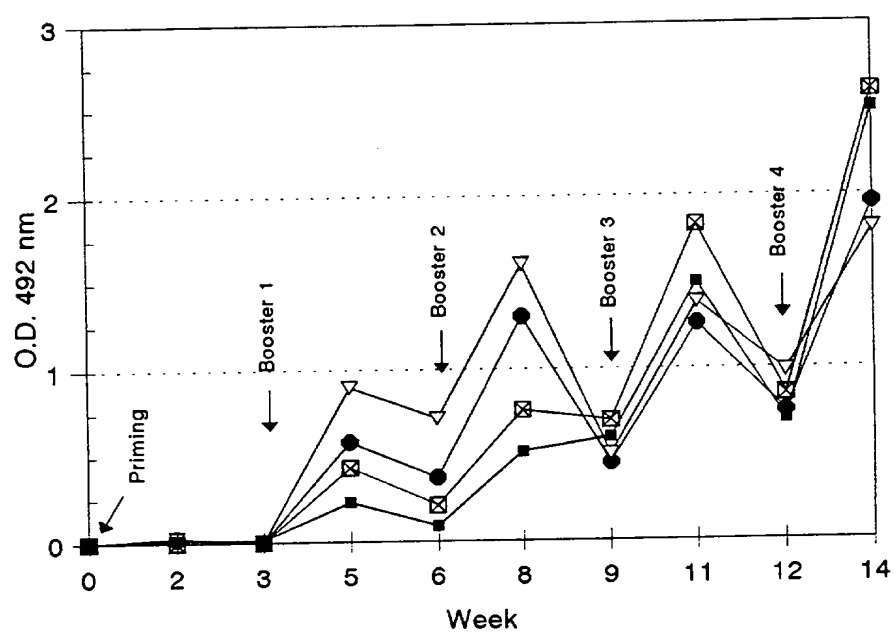
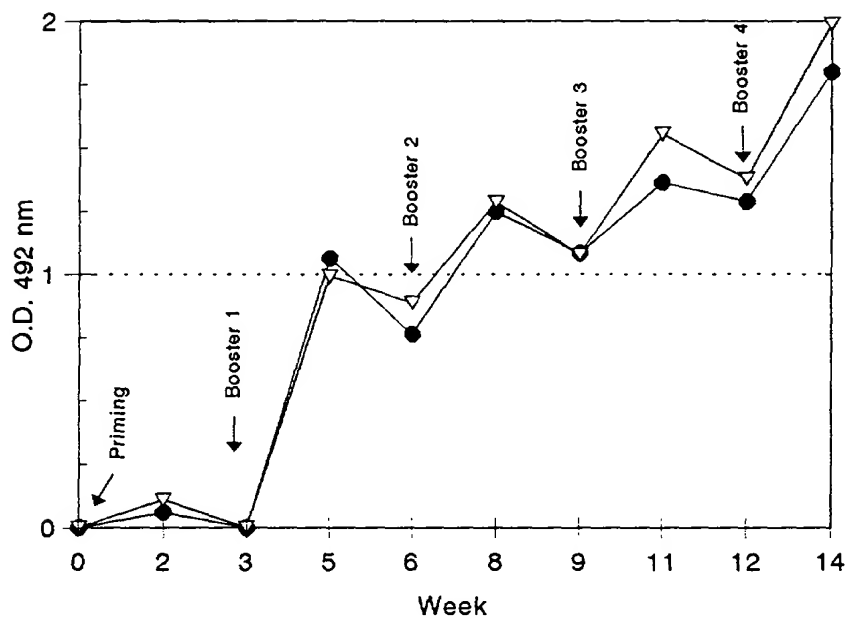


Fig 1



A

Fig 2a



B

Fig 2b

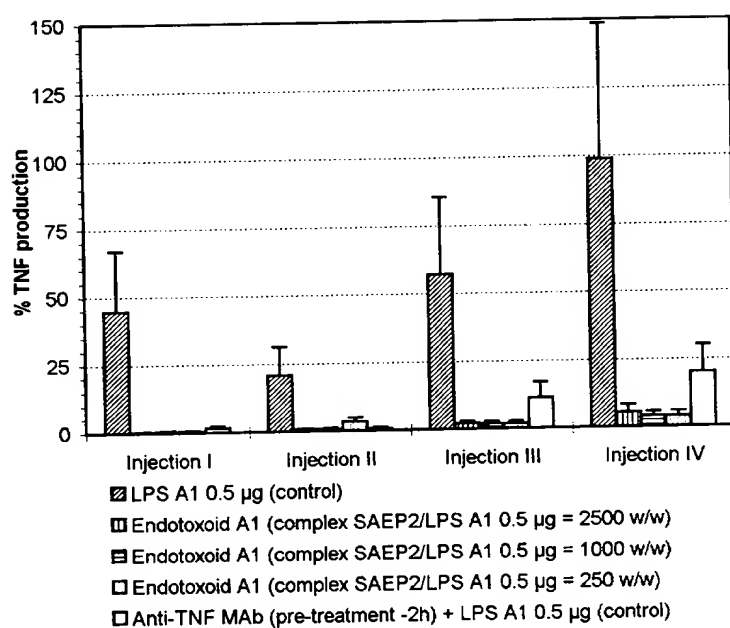


Fig 3

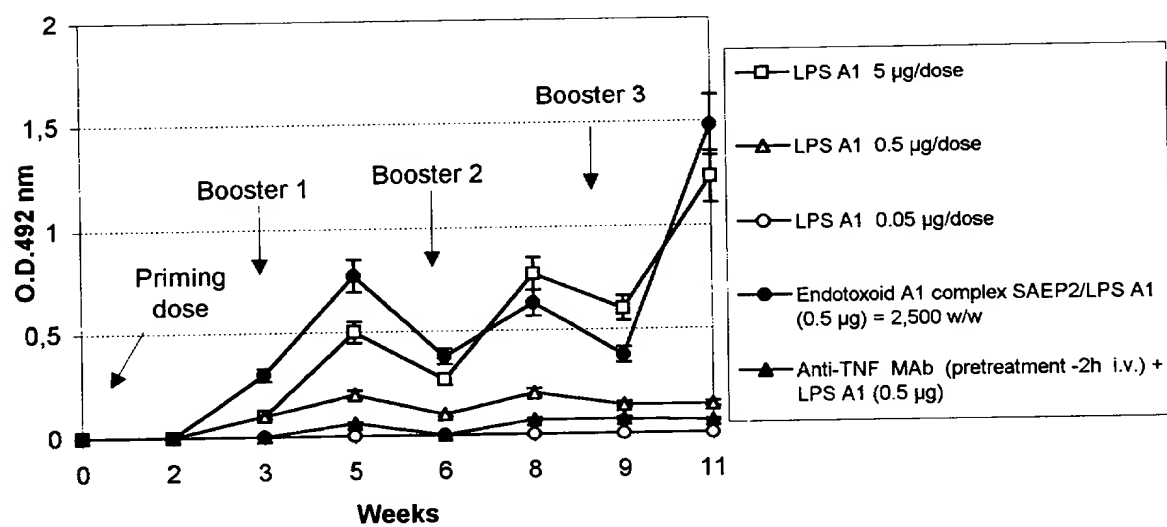


Fig 4a

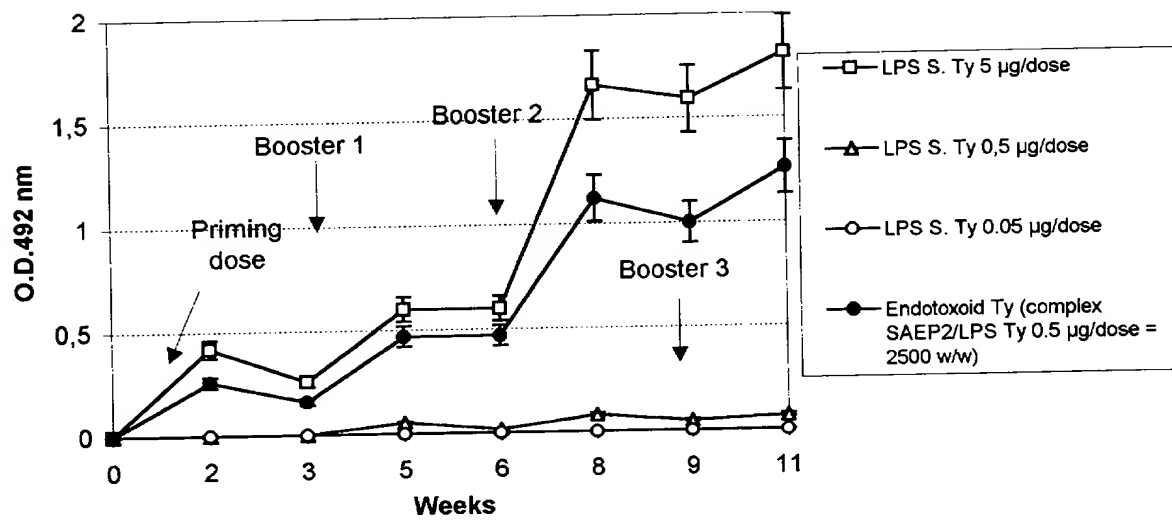
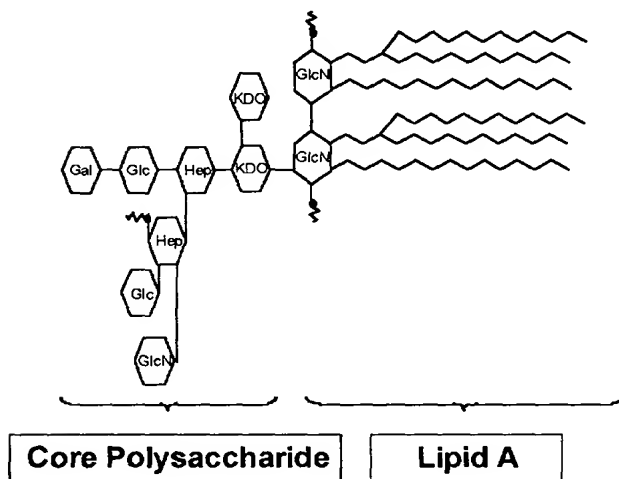
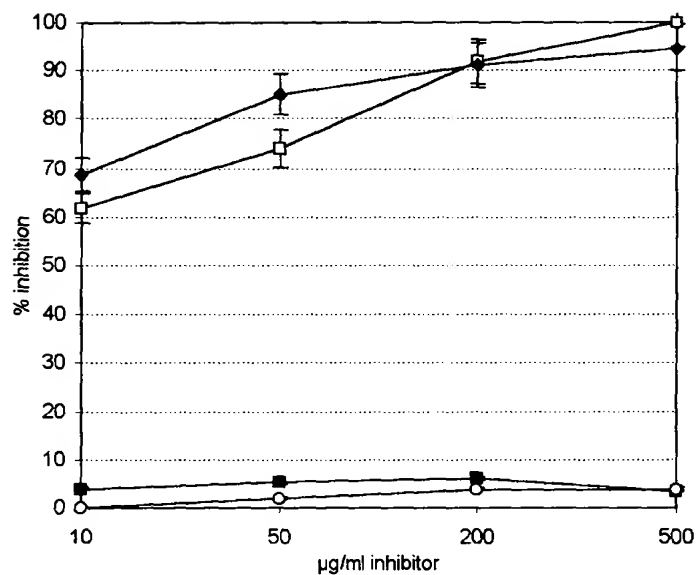


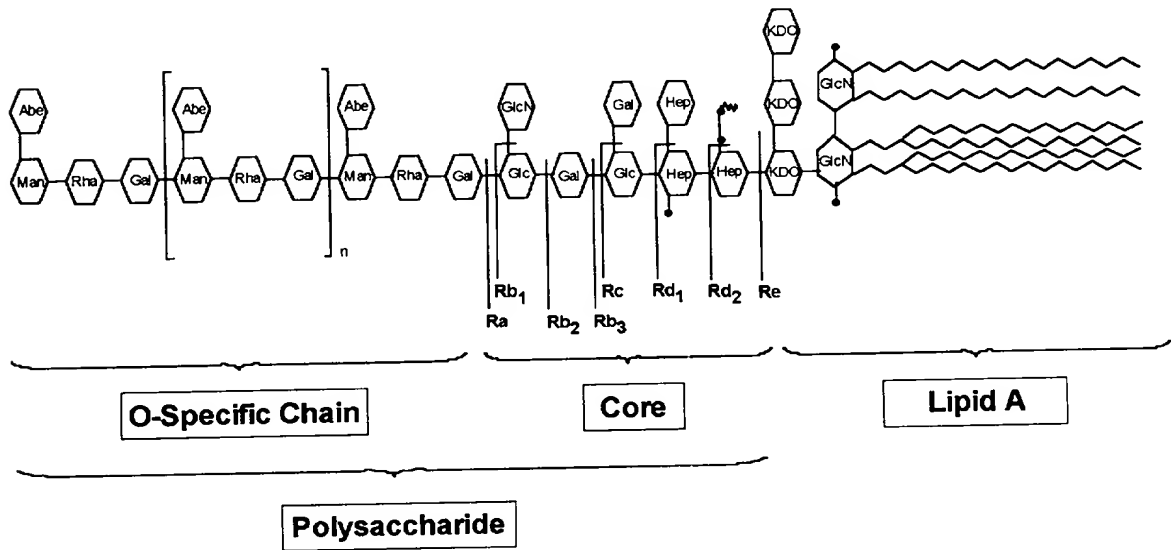
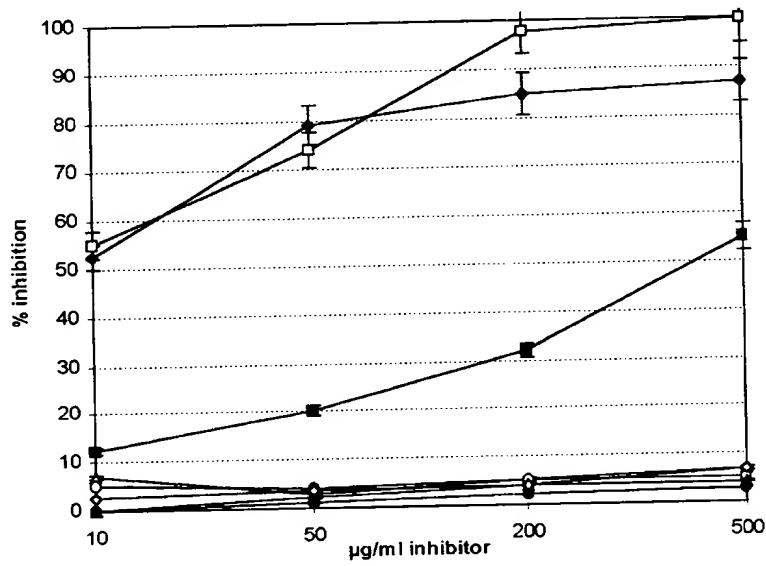
Fig 4b



Schematic representation of the immunochemical specificity of mouse IgG polyclonal antibodies induced by endotoxin A1. Analysis was performed by inhibition ELISA, at the serum dil. 1:800 (v/v) using homologous and heterologous LPS. ◆ N. meningitidis group A strain A1 LPS ; □ N. meningitidis group B strain BB431 LPS ; ■ S. minnesota LPS Re chemotype ; ○ E. coli 055:B5 LPS.

⬡ : Monosaccharide ; • Phosphate ; ~ : Ethanolamin ; ~ : Long Chain (Hydroxy) Fatty Acid.

Fig 5a

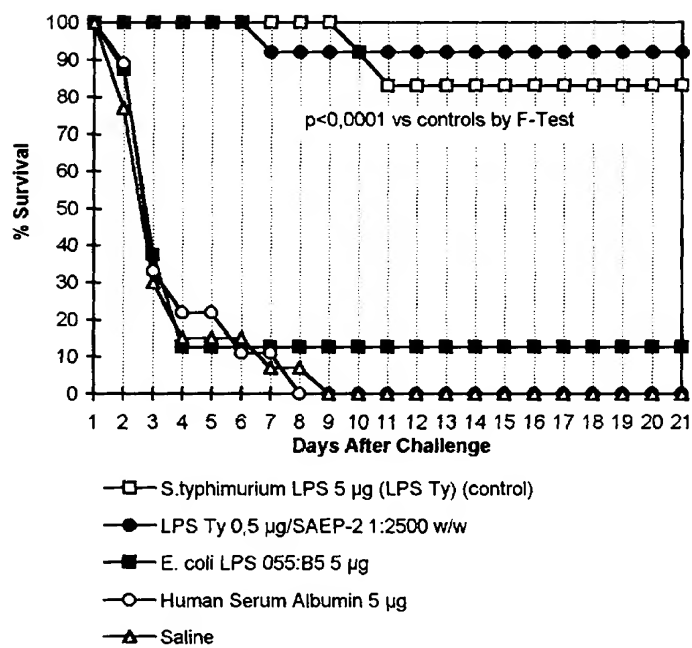


Schematic representation of the Immunochemical specificity of mouse IgG polyclonal antibodies induced by the endotoxin Ty. Analysis was performed by inhibition ELISA, at the serum dil. 1:1,000 (v/v) using different LPS chemotypes from *S. typhimurium*. □ *S. enterica* LPS ; ◆ *S. typhosa* LPS ; ■ *S. typhimurium* LPS Ra chemotype ; △ *S. typhimurium* LPS Rb1 chemotype ; ○ *S. typhimurium* LPS Rc chemotype ; ▲ *S. typhimurium* LPS Rd2 chemotype ; ● *S. typhimurium* LPS Re chemotype ; ◇ SAEP2.

⬡ : Monosaccharide ; • Phosphate ; ~ : Ethanolamin ; ~~~~ : Long Chain (Hydroxy) Fatty Acid.

Fig 5b

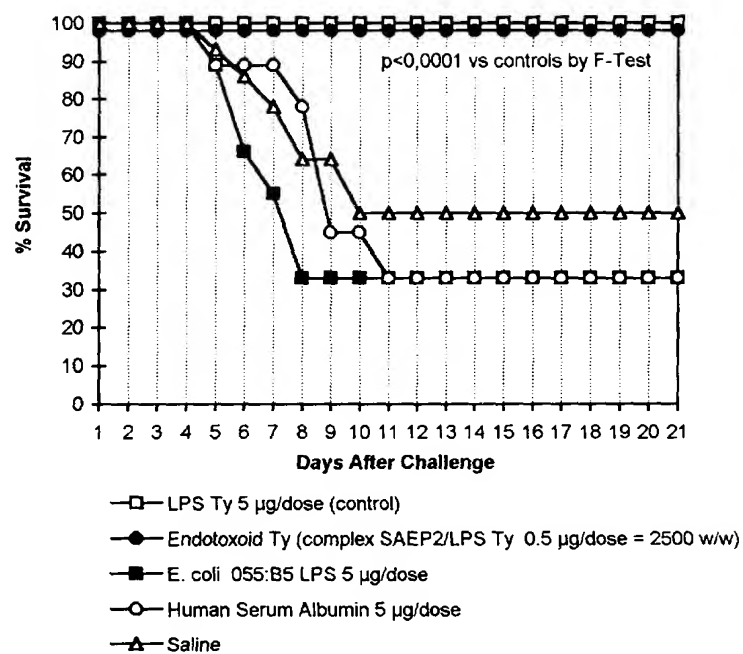
$LD_{100} = 4 \times 10^5$ cells/dose



Average number of CD1 mice/group = 12

Fig 6a

$LD_{50} = 9 \times 10^3$ cells/dose



Average number of CD1 mice/group = 12

Fig 6b

SMALL BUSINESS

Docket No.: 575-808

Applicant(s) or Patentee(s): Massimo Porro

Examiner: -

Serial or Patent No.: Filed or Issued: Art Unit: -

For: VACCINE FOR PREVENTION OF GRAM-NEGATIVE BACTERIAL INFECTIONS AND ENDOTOXIN RELATED DISEASES

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR § 1.9(F) AND § 1.27(C)) - SMALL BUSINESS CONCERN

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act
on behalf of the concern identified below:

NAME OF CONCERN: BioSynth S.r.l.
ADDRESS: Zona Industriale-Loc Sentino
53040 Rapolano Terme, Siena, Italy

I hereby declare that the above-identified small business qualifies as a small business concern as defined in 13 CFR §121.3-18, and reproduced in 37 CFR §1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, DOES NOT EXCEED 500 PERSONS. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled VACCINE FOR PREVENTION OF GRAM-NEGATIVE BACTERIAL INFECTIONS AND ENDOTOXIN RELATED DISEASES,

by inventor(s): Massimo Porro
described in:

- ☒ the specification filed herewith
☐ application serial no. _____, filed _____
☐ patent no. _____, issued _____

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR §1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR §1.9(d) or a nonprofit organization under 37 CFR §1.9(e).

NAME: _____

ADDRESS: _____

- ☐ INDIVIDUAL
☐ SMALL BUSINESS CONCERN
☐ NONPROFIT ORGANIZATION
☐ See attached sheet for additional person(s), concern(s), or organization(s).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR §1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, my patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: _____ TITLE: _____
ADDRESS: _____

SIGNATURE:  DATE: 07/23/98

NOTE: SEPARATE VERIFIED STATEMENTS ARE REQUIRED FROM EACH NAMED PERSON, CONCERN OR ORGANIZATION HAVING RIGHTS TO THE INVENTION AVERRING TO THEIR STATUS AS SMALL ENTITIES (37 CFR §1.27)

0824160
072998

Docket No: 576-008

**APPLICATION FOR UNITED STATES LETTERS PATENT
DECLARATION, POWER OF ATTORNEY, AND PETITION**

As a below-named inventor, I declare that:

My residence, post office address and citizenship are as stated next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the invention which is described and which is claimed in the specification, entitled: VACCINE FOR PREVENTION OF GRAM-NEGATIVE BACTERIAL INFECTIONS AND ENDOTOXIN RELATED DISEASES.

The specification [x] is attached hereto [] was filed on _____, as Application Serial No. _____.

I hereby state that I have reviewed and understand the contents of said specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §112 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.¹

| COUNTRY | APPLICATION NUMBER | DATE (Day, Month, Year) | PRIORITY CLAIMED UNDER 35 U.S.C. 119 |
|---------|-----------------------|----------------------------|---|
| | | | Yes [] No [] |
| | | | Yes [] No [] |

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

¹In Non-Convention cases, a listing of all filings and current status of cases filed more than a year before the U.S. filing is required to comply with 37 CFR 1.56(a). Such a listing may be attached.

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-2-


| APPLICATION SERIAL NO. | FILING DATE | STATUS |
|------------------------|-------------|--------|
| | | |
| | | |

I hereby appoint my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the U.S. Patent & Trademark Office connected therewith:

Edward A. Hedman, Reg. No. 22,120; Thomas M. Gibson, Reg. No. 24,638; James V. Costigan, Reg. No. 25,669; Kenneth F. Florek, Reg. No. 33,173; Alan B. Clement, Reg. No. 34,563; Martin P. Endres, Reg. No. 35,498 and Timothy X. Gibson, Reg. No. 40,618.

CORRESPONDENCE AND CALLS TO: James V. Costigan, Esq.
HEDMAN, GIBSON & COSTIGAN, P.C.
1185 Avenue of the Americas
New York, NY 10036-2601
Telephone: (212) 302-8989

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

| INVENTOR(S) | DATE | RESIDENCE AND P.O. ADDRESS |
|---|-------------------------------------|---|
| Name: Massimo Porro Signature:  | Date: 07/23/98 Citizen of: Italy | Via Selvapina, 97 53040 Rapolano Terme Siena, Italy |
| Name: Signature: | Date: Citizen of: | |
| Name: Signature: | Date: Citizen of: | |
| Name: Signature: | Date: Citizen of: | |

RECEIVED 07/23/98